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13. ABSTRACT (Maximum 200 words) This AASERT grant, together with supplementary funding from the Texas Agricultural Experiment Station, provided support between September 1993 and August 1996 for the dissertation research of several predoctoral students. Highlights of their collective accomplishments include: a) participating in the crystallization and analysis of the high resolution structures of the bacterial luciferase $\alpha\beta$ heterodimer and the β_2 homodimer, b) elucidation of a detailed kinetic mechanism for the folding and assembly of the luciferase subunits, c) use of spectroscopic techniques and mutant enzymes to probe the active site and subunit interface of bacterial luciferase, and d) characterization by genetic methods of the role of the LuxR transcriptional activator protein in density-dependent control of gene expression. The studies of the students who were supported by this grant represent a highly productive element of our ONR-supported investigations of structure-function relationships in bioluminescent proteins. Furthermore, the students themselves represent well-educated scientists whose predoctoral training has prepared them to pursue productive careers in research.					
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FINAL TECHNICAL REPORT

OFFICE OF NAVAL RESEARCH

ONR Grant #

N00014-93-1-1345

Grant Title

**Structural and Functional Studies of
Bioluminescent Proteins**

Grant Period

1 September 1993 - 31 August 1996

Principal Investigators

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ACCOMPLISHMENTS: This AASERT grant, together with supplementary funding from the Texas Agricultural Experiment Station, provided support between September 1993 and August 1996 for the dissertation research of several predoctoral students, including A. Clay Clark, James F. Sinclair, Dmitry M. Sitnikov, Stephen W. Raso, and Jon A. Christopher, studying structure and function of bioluminescent proteins. Highlights of their collective accomplishments are outlined below.

Crystallization conditions for the bacterial luciferase heterodimer ($\alpha\beta$) were refined, with the resultant crystals permitting high resolution data to be collected and interpreted. In collaboration with Dr. Ivan Rayment of the University of Wisconsin, data were collected and analyzed from one crystal form at 2.4 Å resolution (refereed ref. 7 below) and ultimately from another crystal form at 1.5 Å resolution (refereed ref. 13 below). Crystallization protocols were also developed for mutant luciferases and the β_2 homodimer that forms when the β subunit is expressed without α in *E. coli*; the homodimer structure was solved at 1.95 Å resolution, again in collaboration with the laboratories of Drs. Ivan Rayment and Hazel Holden (refereed ref. 15 below).

Clay Clark characterized two types of mutant luciferases. One group of mutants comprises temperature-sensitive folding mutants with various substitutions at position 313 of the β subunit. The β 313 mutations affect the rate of folding of the enzyme, but not the stability of the finally folded structure (refereed ref. 17 below, in preparation). The other set of mutants each has one of the wild-type Trp residues replaced by Phe or Leu, in an effort to identify aromatic side chains potentially involved in stacking interactions with the flavin substrate. Two of the mutant enzymes showed marked changes in catalytic parameters, and thus represent possible active site mutants (A. C. Clark, Ph.D. thesis, 1995). Dr. Clark and another student, Stephen Raso, also carried out a careful analysis of all the data that had been collected for the kinetics of folding and assembly of the heterodimer and of the individual subunits, arriving at a detailed kinetic mechanism for folding (refereed ref. 16 below). Dr. Clark received his Ph.D. in August 1994, and is currently a postdoctoral fellow in the laboratory of Dr. Carl Frieden at Washington University in St. Louis.

James Sinclair characterized the different conformational forms of the β subunit, including the β_2 species, obtained by folding of the protein under different conditions (in the presence or absence of the α subunit, at different temperatures, different pH values, different ionic strengths, etc.). He demonstrated definitively that the finally folded structure of the β polypeptide is determined by kinetic factors rather than by the stability of the finally folded product (refereed refs. 4 and 6 below). Dr. Sinclair also was instrumental in obtaining the three-dimensional structure of the β_2 homodimer, having obtained the first crystals of that species (refereed ref. 15 below). He received his Ph.D. in May 1995 and is currently a postdoctoral fellow in the laboratory of Dr. David Shortle at the Johns Hopkins University.

Dmitry Sitnikov characterized the functional domains of LuxR, the transcriptional activator protein from the bioluminescent bacterium *Vibrio fischeri* (refereed refs. 8, 10 and 14 below). This protein participates in a regulatory system involving cell density-dependent control of gene expression ("quorum sensing"), mediated by a small

molecule autoinducer. The *V. fischeri* system has turned out to be the paradigm for cell density-dependent control of gene expression in many bacterial species (see ref. 8 below). Using mutational analysis, Dr. Sitnikov provided genetic evidence for direct interactions of LuxR with RNA polymerase as well as with DNA. The results of his genetic construction of plasmids encoding fusion proteins involving either an N-terminal "leucine zipper" domain or an N-terminal AraC domain with the C-terminal transcriptional activator domain of LuxR suggest that LuxR functions as an oligomer, possibly as a dimer (Sitnikov, Ph.D. thesis, 1996). Dr. Sitnikov received his Ph.D. in August 1996 and is currently a postdoctoral fellow in the laboratory of Dr. Andrew Wright at Tufts University.

Stephen Raso has investigated the pathway of luciferase subunit folding and assembly using two different approaches: 1) a combination of spectroscopic methods and enzymatic activity for monitoring the kinetics of luciferase refolding, and 2) subunit complementation methods, in which he has used inactive mutants of luciferase and simulated the process of subunit exchange using the folding mechanism he helped to develop (refereed reference 16 below). He has also used spectroscopic methods (e.g. circular dichroism spectroscopy of the enzyme-bound 4a-peroxyflavin intermediate) to probe structure-function relationships in the luciferase active site, using not only the wild-type enzyme but also active site mutants such as AK-6, which is of particular interest because it has an altered bioluminescence emission spectrum. Mr. Raso expects to finish his Ph.D. dissertation in August or September 1997, and plans to pursue postdoctoral training at M.I.T. in the laboratory of Dr. Jonathan King.

Jon Christopher has approached protein folding questions using computational methods. He performed a statistical survey of the distance between the N- and C-termini of known structures in the Protein Database with an emphasis on the effects of terminal distance on the protein folding pathway (refereed ref. 12 below), and developed helical axis calculation methods along the way (refereed ref. 9 below). He was also instrumental in locating the putative active site in bacterial luciferase by structural, electrostatic and surface modeling studies (ref. 5 under reviews, below). More recently he has been working on computer-assisted detection of low-barrier hydrogen bonds in protein structures. Mr. Christopher plans to finish his Ph.D. dissertation during the fall of 1997.

SIGNIFICANCE: The studies of the students who were supported by this grant represent a highly productive element of our investigations of structure-function relationships in bioluminescent proteins. The three-dimensional structure of bacterial luciferase and the mechanism of the folding and assembly of its subunits are of fundamental importance to the long-term goals of understanding the catalytic mechanism and the mode of interaction of the enzyme with accessory proteins; the structure of the heterodimer and that of the β_2 homodimer have suggested new avenues of investigation in pursuit of those goals. Well-characterized mutants of bacterial luciferase have been extremely useful in our efforts to characterize the active site in the three-dimensional structure of the wild-type enzyme, and represent a major contribution toward our objective of defining the roles of specific amino acid residues in substrate binding and catalysis and in protein-protein interactions. Furthermore, the

students themselves represent well-educated scientists whose predoctoral training has prepared them to pursue productive careers in research.

PUBLICATIONS RELATED TO THIS GRANT (1993-1997)

Ph.D. Dissertations of Students Supported by the AASERT Grant

1. Clark, A. C. (1994) Thermodynamic and kinetic studies of the polypeptide folding of bacterial luciferase from *Vibrio harveyi*: a mutational analysis. Ph.D. thesis, Texas A&M University, College Station, TX.
2. Sinclair, J. F. (1995) Equilibrium and kinetic studies of the folding of the subunits of bacterial luciferase. Ph.D. thesis, Texas A&M University, College Station, TX.
3. Sitnikov, D. M. (1996) Genetic and physiological studies of the *Vibrio fischeri lux* regulon. Ph.D. thesis, Texas A&M University, College Station, TX.
4. Raso, S. W. (1997) The use of mutant enzymes to probe the structure, function and folding pathway of bacterial luciferase. Ph.D. thesis, projected completion August or September 1997, Texas A&M University, College Station, TX.
5. Christopher, J. A. (1997) Computer-assisted detection of low-barrier hydrogen bonds in protein structures. Ph.D. thesis, projected completion fall 1997, Texas A&M University, College Station, TX.

Refereed Publications

1. Ziegler, M. M., Goldberg, M. E., Chaffotte, A. F. and Baldwin, T. O. (1993) Refolding of luciferase subunits from urea and assembly of the active heterodimer. Evidence for folding intermediates that precede and follow the dimerization step on the pathway to the active form of the enzyme. *J. Biol. Chem.* **268**, 10760-10765.
2. Baldwin, T. O., Ziegler, M. M., Chaffotte, A. F. and Goldberg, M. E. (1993) Contribution of folding steps involving the individual subunits of bacterial luciferase to the assembly of the active heterodimeric enzyme. *J. Biol. Chem.* **268**, 10766-10772.
3. Clark, A. C., Sinclair, J. F. and Baldwin, T. O. (1993) Folding of bacterial luciferase involves a non-native heterodimeric intermediate in equilibrium with the native enzyme and the unfolded subunits. *J. Biol. Chem.* **268**, 10773-10779.
4. Sinclair, J. F., Waddle, J. J., Waddill, E. F. and Baldwin, T. O. (1993) Purified native subunits of bacterial luciferase are active in the bioluminescence reaction but fail to assemble into the $\alpha\beta$ structure. *Biochemistry* **32**, 5036-5044.
5. Abu-Soud, H., Clark, A. C., Francisco, W. A., Baldwin, T. O. and Raushel, F. M. (1993) Kinetic destabilization of the hydroxyperoxyflavin intermediate by site-directed modification of the reactive thiol in bacterial luciferase. *J. Biol. Chem.* **268**, 7699-7706.
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7. Fisher, A. J., Raushel, F. M., Baldwin, T. O. and Rayment, I. (1995) The three-dimensional structure of bacterial luciferase from *Vibrio harveyi* at 2.4 Å resolution. *Biochemistry* **34**, 6581-6586.
8. Sitnikov, D. M., Schineller, J. B. and Baldwin, T. O. (1995) Transcriptional regulation of bioluminescence genes from *Vibrio fischeri*. *Molecular Microbiology* **17**, 801-812.
9. Christopher, J. A., Swanson, R. F. and Baldwin, T. O. (1996) Algorithms for finding the axis of a helix: Fast rotational and parametric least-squares methods. *Computers and Chemistry* **20**, 339-345.
10. Sitnikov, D. M., Schineller, J. B. and Baldwin, T. O. (1996) Control of cell division in *Escherichia coli*: Regulation of transcription of *ftsQA* involves both *rpoS* and SdiA-mediated autoinduction. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 336-341.
11. Xia, J., Sinclair, J. F., Baldwin, T. O. and Lindahl, P. A. (1996) CO dehydrogenase from *Clostridium thermoaceticum*: Quaternary structure, stoichiometry of its SDS-induced dissociation, and characterization of the faster-migrating form. *Biochemistry* **35**, 1965-1971.
12. Christopher, J. A. and Baldwin, T. O. (1996) Implications of N- and C-terminal proximity for protein folding. *J. Mol. Biol.* **257**, 175-187.
13. Fisher, A. J., Thompson, T. B., Thoden, J. B., Baldwin, T. O. and Rayment, I. (1996) The 1.5 Å resolution crystal structure of bacterial luciferase in low salt conditions. *J. Biol. Chem.* **271**, 21956-21968.
14. Sitnikov, D. M., Shadel, G. S. and Baldwin, T. O. (1996) Autoinducer-independent mutants of the LuxR transcriptional activator exhibit differential effects on the two *lux* promoters of *Vibrio fischeri*. *Mol. Gen. Genet.* **252**, 622-625.
15. Thoden, J. B., Holden, H. M., Fisher, A. J., Sinclair, J. F., Wesenberg, G., Baldwin, T. O. and Rayment, I. (1997) X-ray structure of the β -homodimer of bacterial luciferase from *Vibrio harveyi* to 1.95 Å resolution. *Protein Science* **6**, 13-23. (Data were collected during the period of support of the AASERT grant, which was acknowledged.)
16. Clark, A. C., Raso, S. W., Sinclair, J. F., Ziegler, M. M., Chaffotte, A. F. and Baldwin, T. O. (1997) Kinetic mechanism of luciferase subunit folding and assembly. *Biochemistry* **36**, 1891-1899. (Data were collected during the period of support of the AASERT grant, which was acknowledged.)
17. Clark, A. C., Waddill, E. F., Chaffotte, A. F. and Baldwin, T. O. (1997) Kinetic control of protein folding: mutational analysis of alternative folding pathways. (manuscript in preparation) (Data were collected during the period of support of the AASERT grant, which is being acknowledged.)

Reviews, Book Chapters and Symposium Contributions

1. Ziegler, M. M., Clark, A. C., Sinclair, J. F., Chaffotte, A. F., Goldberg, M. E. and Baldwin, T. O. (1993) Folding and assembly of the subunits of bacterial luciferase. In *Bioluminescence and Chemiluminescence*, eds. A. A. Szalay, L. J. Kricka, and P. Stanley (John Wiley & Sons) pp. 178-182.

2. Baldwin, T. O., Ziegler, M. M., Sinclair, J. F., Clark, A. C., Chaffotte, A.-F. and Goldberg, M. E. (1994) Mechanism of folding and assembly of heterodimeric bacterial luciferase. In Flavins and Flavoproteins 1993, ed. K. Yagi (Walter de Gruyter, Berlin) pp. 823-828.
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4. Christopher, J. A., Raso, S. W., Ziegler, M. M. and Baldwin, T. O. (1995) Enzymes. In Molecular Biology and Biotechnology: A Comprehensive Desk Reference, ed. R. A. Meyers (VCH Publishers, Weinheim, New York, Basel and Cambridge) pp. 295-301.
5. Baldwin, T. O., Christopher, J. A., Raushel, F. M., Sinclair, J. F., Ziegler, M. M., Fisher, A. J., and Rayment, I. (1995) Structure of bacterial luciferase. *Curr. Opin. Struct. Biol.* **5**, 798-809.
6. Raso, S. W., Christopher, J. A., Ziegler, M. M. and Baldwin, T. O. (1996) Enzymes: the energetics and regulation of biological catalysis. In The Encyclopedia of Molecular Biology: Fundamentals and Applications (VCH Publishers, Weinheim, New York, Basel and Cambridge) pp. 222-240.
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8. Baldwin, T. O., Ziegler, M. M., Clark, A. C., Sinclair, J. F., Raushel, F. M., Rayment, I., Holden, H. M., Fisher, A. J., Thompson, T. B. and Thoden, J. B. (1997) Structure and folding of bacterial luciferase. In Flavins and Flavoproteins 1996 (K. J. Stevenson, V. Massey and C. H. Williams, eds.) University of Calgary Press, in press.